5-Fluorouracil-induced apoptosis in colorectal cancer cells is caspase-9-dependent and mediated by activation of protein kinase C-δ

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Abstract. Elucidation of the molecular mechanisms by which 5-fluorouracil (5-FU) induces apoptosis is required in order to understand the resistance of colorectal cancer (CRC) cells to 5-FU. In the current study, 5-FU-induced apoptosis was assessed using the propidium iodide method. Involvement of protein kinase C (PKC) was assessed by evaluating the extent of their activation in CRC, following treatment with 5-FU, using biochemical inhibitors and western blot analysis. The results revealed that 5-FU induces varying degrees of apoptosis in CRC cells; HCT116 cells were identified to be the most sensitive cells and SW480 were the least sensitive. In addition, 5-FU-induced apoptosis was caspase-dependent as it appeared to be initiated by caspase-9. Furthermore, PKCε was marginally expressed in CRC cells and no changes were observed in the levels of cleavage or phosphorylation following treatment with 5-FU. The treatment of HCT116 cells with 5-FU increased the expression, phosphorylation and cleavage of PKCδ. The inhibition of PKCδ was found to significantly inhibit 5-FU-induced apoptosis. These results indicated that 5-FU induces apoptosis in CRC by the activation of PKCδ and caspase-9. In addition, the levels of PKCδ activation may determine the sensitivity of CRC to 5-FU.

Introduction

Colorectal cancer (CRC) is the third most common malignancy in the populations of developed western countries and represents the third leading cause of cancer-associated mortality in the USA (1). Although the incidence of CRC varies by ~20-fold worldwide, in Jordan, CRC is the most common type of cancer in males and the second, following breast cancer, in females (2). Once the disease spreads to distant sites, it is usually incurable using the current systemic treatment options, including chemotherapy. This is largely due to the resistance of cancer cells to apoptosis. Understanding and overcoming the resistance of cancer cells to apoptosis may simplify the identification of novel therapeutic targets and the development of novel treatment options.

The chemotherapeutic agent, 5-fluorouracil (5-FU), induces cytotoxic effects via targeting the metabolism of RNA bases (3). It is predominantly administered in the treatment of various types of cancer, including breast, aerodigestive tract and head cancer. In CRC, 5-FU monopolizes a place of choice even in individuals with advanced metastatic disease (4,5). The response rate associated with 5-FU monotherapy is only 10-15%, however, in a small number of cases the response rate was >50% when associated with other types of medication (6-8).

The protein kinase C (PKC) family includes 11 distinct members, which share a similar serine (Ser)/threonine (Thr) structure (9,10). These polypeptide isoenzymes are classically classified into three distinct groups according to the second messengers to which they respond. Conventional PKCs, PKCα, β1, β2 and γ, may be activated by diacylglycerol (DAG) and calcium (Ca^{2+}). The activation of novel PKCs, PKCδ, θ, η and ε, is DAG-dependent and $Ca2^{+}$-independent. The activation of atypical PKCs, PKCι/λ and ζ, is independent of these second messengers (11). PKCε is an antiapoptotic enzyme, which promotes cell proliferation and resistance to chemotherapeutic agents (12), whereas PKCδ is classified among the proapoptotic PKCs as its cleavage and activation promotes apoptotic cell death (13).

Materials and methods

Cell lines. The human CRC cell lines, SW480, SW620, HCT116 and HT29, were obtained from Dr Rick F. Thorne (University of Newcastle, Newcastle, NSW, Australia) and were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Antibodies and other reagents. 5-FU was obtained from Ebewe Pharma (Unterach, Austria), stored at room

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temperature, dissolved in dimethyl sulfoxide and prepared as a stock solution of 200 μM immediately prior to use. The pan-caspase inhibitor, Z-VAD-fmk; the caspase-2-specific inhibitor, z-VDVAD-fmk; the caspase-3-specific inhibitor, z-DEVAD-fmk; the caspase-8-specific inhibitor, z-IETD-fmk; and the caspase-9-specific inhibitor, z-LEHD-fmk, were purchased from R&D Systems (Minneapolis, MN, USA). The general PKC inhibitor, bisindolylmaleimide I (GF109203X), and the specific PKCδ inhibitor, rottlerin, were purchased from Calbiochem (La Jolla, CA, USA), while the cell-permeable-specific PKCε inhibitor, epsilon-V1-2 inhibitor Cys-conjugated, was obtained from AnaSpec Inc. (Fremont, CA, USA). The mouse anti-human monoclonal antibodies directed against caspase-2, -3, -8 and -9, propidium iodide (PI) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The rabbit polyclonal antibodies, anti-PKCε, p-PKC, PKCδ and p-PKCδ Ser 645, and the mouse monoclonal antibody directed against p-PKCδ, Thr 505, were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell viability analysis. The MTT assay was performed as previously described to assess the viability of the CRC cells following treatment with 5-FU (14).

Apoptosis. The quantification of the apoptotic cells was obtained by measuring the sub-G1 population of apoptotic cells using flow cytometry following staining with PI as previously described (15).

Western blot analysis. Changes in protein expression induced by 5-FU were assessed by western blot analysis as previously described (15).

Statistical analysis. Data are presented as the mean ± standard error. The statistical significance of intergroup differences in normally distributed continuous variables was determined using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

5-FU induces the caspase-dependent apoptosis of human CRC cells. To examine the antitumor potential of 5-FU, cell viability analysis was performed using the MTT assay on a panel of CRC cell lines. The cells were incubated with a wide range of 5-FU concentrations (0, 1, 10, 25, 50, 100 and 200 μM) for 72 h. As shown in Fig. 1, 5-FU induced growth inhibition in a dose-dependent manner in all CRC cells, to different degrees. The optimal concentration was 50 μM. HCT116 cells were the most sensitive cells followed by HT29 and SW620, and the least sensitive cell line was SW480. In addition, kinetic analysis revealed that 5-FU (50 μM) induced a significant growth inhibition following 72 h of incubation (Fig. 2).

To investigate whether the 5-FU-induced reduction in cell viability is due to the induction of apoptotic cell death, the cellular and molecular apoptotic events were evaluated following treatment with 5-FU (50 μM) for 72 h. The results showed different degrees of CRC sensitivity to 5-FU-induced apoptosis. HCT116 represented the most sensitive cell line, HT29 and SW620 were intermediately sensitive, and SW480 cells were the least sensitive (Fig. 3).

To assess the involvement of caspases in 5-FU-induced apoptosis, HCT116 and SW480 cells were pretreated with the

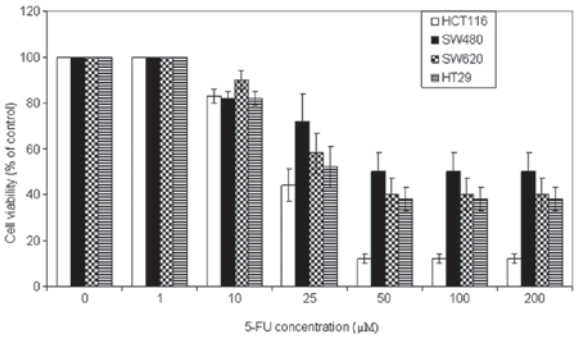

Figure 1. 5-FU inhibits CRC cell growth. HCT116, SW480, HT29 and SW620 human CRC cell lines were treated with a wide range of 5-FU prior to assess cell viability using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Data are presented as the mean ± standard error of three individual experiments. 5-FU, 5-fluorouracil; CRC, colorectal cancer.

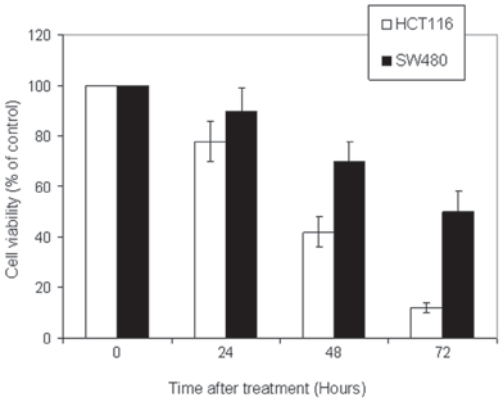

Figure 2. 5-Fluorouracil (FU) induces time-dependent cell growth inhibition in colorectal cancer cells. HCT116 and SW480 cells were treated with 5-FU (50 μM) for different time periods. The cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Data are representative of three individual experiments.

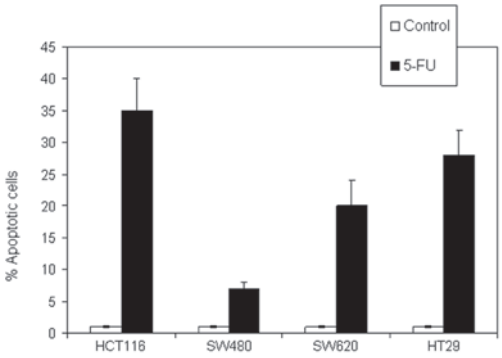

Figure 3. 5-FU-induced apoptosis in a panel of human CRC cell lines. CRC cells were treated with 5-FU (50 μM) for 72 h. The apoptotic cell fractions were determined using the propidium iodide method. Data are representative of three independent experiments. 5-FU, 5-fluorouracil; CRC, colorectal cancer.

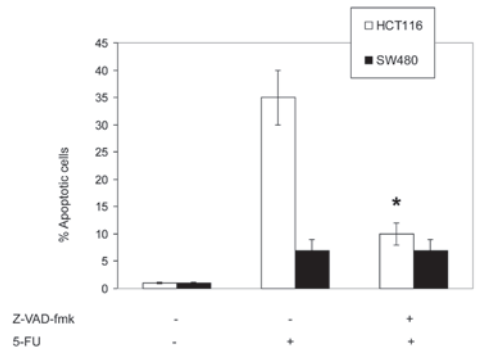

Figure 4. 5-Fluorouracil (FU) induces caspase-dependent apoptosis. HCT116 and SW480 cells were pretreated with the pan-caspase inhibitor, Z-VAD-fmk (20 μM), for 2 h prior to the addition of 5-FU for an additional 72 h. Apoptosis was measured using the propidium iodide method using flow cytometry. Data are presented as the mean ± standard error of three individual experiments. *P<0.05 vs. 5-FU treatment group.

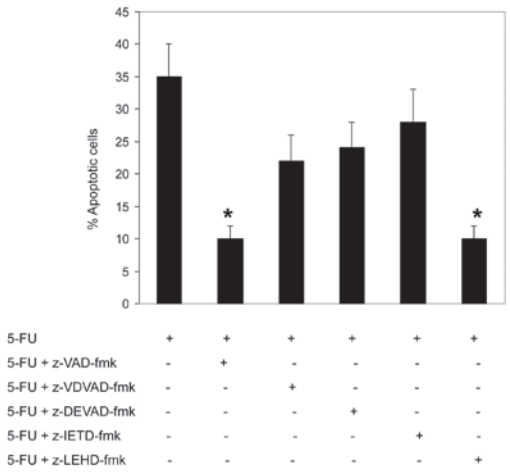

Figure 5. Effect of specific caspase inhibitors on 5-FU-induced apoptosis. HCT116 cells were pretreated with the pan-caspase inhibitor, z-VAD-fmk (20 μM); the caspase-2 inhibitor, z-VDVAD-fmk (30 μM); the caspase-3 inhibitor, z-DEVAD-fmk (30 μM); the caspase-8 inhibitor, z-IETD-fmk (30 μM); and the caspase-9 inhibitor, z-LEHD-fmk (30 μM) 2 h prior to the addition of 5-FU for an additional 72 h. Apoptosis was measured using the propidium iodide method using flow cytometry. Data are presented as the mean ± standard error of three individual experiments. *P<0.05 vs. 5-FU treatment group. 5-FU, 5-fluorouracil.

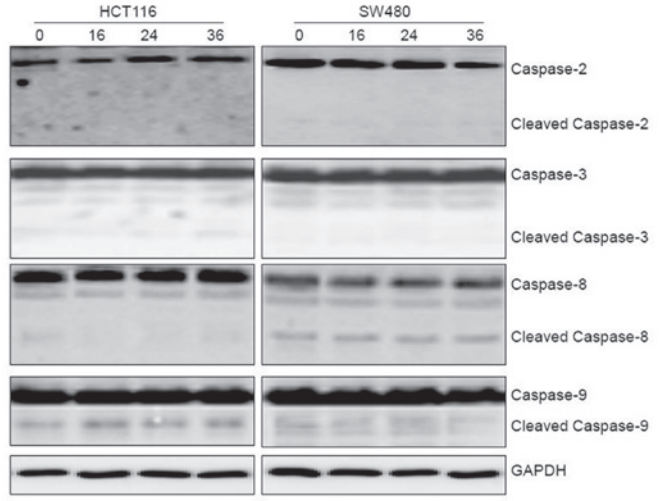

Figure 6. 5-FU induces caspase-9-dependent apoptosis. HCT116 and SW480 cells were treated with 5-FU (50 μM) for the indicated time points (0, 16, 24 and 36 h). Whole cell lysates were subjected to western blot analysis and western blot analysis of the GAPDH levels was included to demonstrate that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. 5-FU, 5-fluorouracil; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

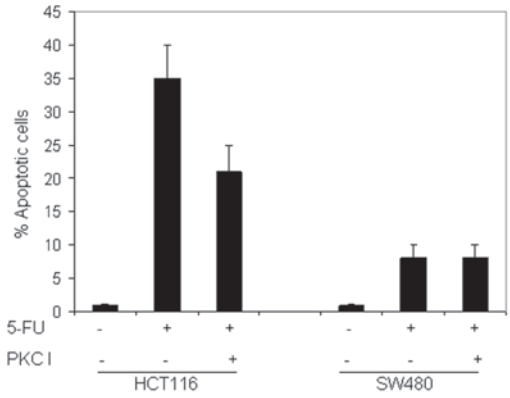

Figure 7. Effect of PKC inhibition on 5-FU-induced apoptosis. HCT116 and SW480 cells treated with 5-FU were pretreated with or without PKC I, bisindolylmaleimide I (5 μM), 2 h prior to the addition of 5-FU (50 μM) for an additional 72 h. Apoptosis was measured using the propidium iodide method using flow cytometry. Data are presented as the mean ± standard error of three individual experiments. 5-FU, 5-fluorouracilPKC, protein kinase C; I, inhibitor.

pan-caspase inhibitor, Z-VAD-fmk (20 μM), and the specific inhibitors of caspase-2, z-VDVAD-fmk (30 μM); caspase-3, z-DEVAD-fmk (30 μM); caspase-8, z-IETD-fmk (30 μM); and caspase-9, Z-LEHD-fmk (30 μM). The inhibitors were added 2 h prior to the addition of 5-FU (50 μM) for an additional 72 h.

As shown in Fig. 4, the pretreatment with the pan-caspase inhibitor, Z-VAD-fmk, was found to significantly inhibit the 5-FU-induced apoptosis in HCT116 cells, which indicates that 5-FU induces a caspase-dependent apoptosis. In addition, these results indicate that the 5-FU-induced apoptosis in HCT116 cells was also significantly inhibited by the specific caspase-9 inhibitor, Z-LEHD-fmk. The inhibition of caspase-2, -3 and -8 exhibited a minimal effect on 5-FU-induced apoptosis.

The kinetics of caspase activation by 5-FU was investigated by western blot analysis (Fig. 6). The cleavage of caspase-9 was evident at 16 h in the HCT116 cells, while the cleavage of the other caspases was not detected in the HCT116 or the SW480 cell lines. These findings demonstrated that caspase-9 is the initial caspase in 5-FU-induced apoptosis.

*5-FU-induced apoptosis is mediated by PKC*δ. To assess the involvement of PKC activation in 5-FU-induced apoptosis, the HCT116 and SW480 cell lines were pretreated with the pan-PKC inhibitor, bisindolylmaleimide I (2.5 or 5 μM; data not shown for 2.5 μM), the specific PKCε inhibitor, epsilon-V1-2 inhibitor cys-conjugated (10 μM), and the specific PKCδ inhibitor, rottlerin (5 μM). The inhibitors were added 2 h prior to the addition of 5-FU for an additional 72 h.

As shown in Fig. 7, pretreatment with the pan-PKC inhibitor reduced the 5-FU-induced apoptosis in HCT116 and

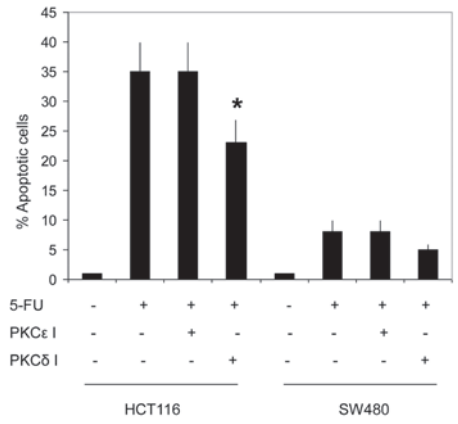

Figure 8. Effect of PKCδ and PKCε inhibition on 5-FU-induced apoptosis. HCT116 and SW480 cells were pretreated with PKCεI, epsilon-V1-2 inhibitor cys-conjugated (10 μM), or PKCδI, rottlerin (5 μM), 2 h prior to the addition of 5-FU for an additional 72 h. The apoptotic percentage was measured using the propidium iodide method. Data are presented as the mean ± standard error of three individual experiments. *P<0.05 vs. 5-FU treatment group. 5-FU, 5-fluorouracilPKC, protein kinase C; I, inhibitor.

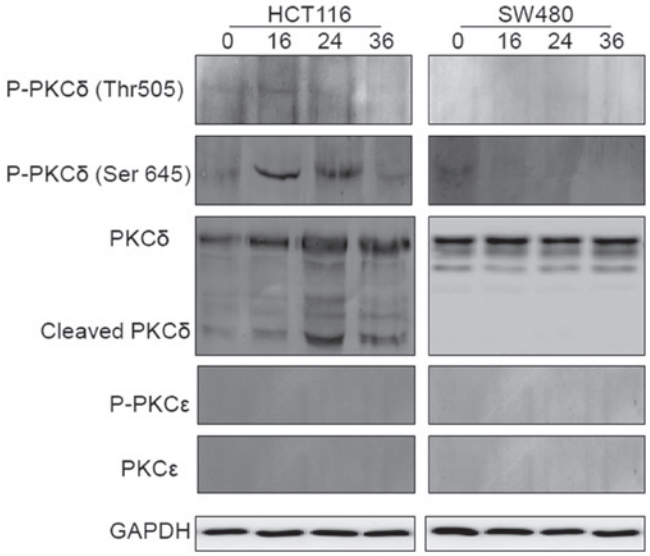

Figure 9. 5-FU induces PKCδ activation in the HCT116 cell line. HCT116 and SW480 cells were treated with 5-FU (50 μM) for the indicated time points (0, 16, 24 and 36 h). Whole cell lysates were subjected to western blot analysis and western blot analysis of the GAPDH levels was included to demonstrate that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. 5-FU, 5-fluorouracil; PKC, protein kinase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

did not affect the response in SW480. This may indicate that the PKC pathway is activated by 5-FU in CRC cells, however, does not provide information on the implicated isoform(s). The pretreatment of the two cell lines with the specific PKCε inhibitor did not sensitize the cells to the 5-FU-induced apoptosis. By contrast, the pretreatment of HCT116 with rottlerin significantly inhibited 5-FU-induced apoptosis in HCT116 cells (Fig. 8). In addition, the kinetics of PKCε and PKCδ activation in HCT116 and SW480 cells prior to and following exposure to 5-FU were investigated. The results shown in Fig. 9 demonstrate that the full-length PKCε was particularly small in the two cell lines prior to and following treatment, and did not undergo cleavage or phosphorylation following treatment with 5-FU. Additionally, it appeared that the full-length PKCδ was gradually upregulated and concomitantly cleaved to its active form following exposure to 5-FU in HCT116, although not in SW480. In the sensitive cells, the phosphorylation of PKCδ at Thr 505 preceded that which occurred at Ser 645 and was downregulated. Taken together, these results may indicate that PKCε is not involved in the resistance of CRC cells to 5-FU and that 5-FU induces PKCδ activation in CRC cells.

Discussion

In the present study, molecular and biochemical experiments were conducted to evaluate the *in vitro* cytotoxic effect of 5-FU against a panel of CRC cells. The cytotoxicity of 5-FU appeared to be due to the induction of apoptosis, which was identified by the PI assay. The results revealed that HCT116 was the most sensitive cell line followed by HT29 and SW620, while SW480 cells were the least sensitive.

The caspases, a family of cysteine proteases, are major mediators of the execution phase of apoptosis; possibly by direct activation of the death receptor or following mitochondrial changes (16,17). Caspase-9 and -8 are generally considered to be the initiator caspases in chemotherapy-induced apoptosis. Caspase-2 is unique in the family of caspases as it is the only caspase that may be involved in the initiation and execution of apoptosis (18-20). Thus, various studies have indicated that it is caspase-2, and not caspase-9, that initiates the DNA damage-induced apoptosis (19,21,22).

In the present study, kinetic analysis of caspase cascade activation using western blot analysis with specific caspase inhibitors revealed that the activation of caspase-9 is the initiating event, which establishes caspase-9 as the apical caspase in the 5-FU-induced apoptosis. This was consistent with previous reports indicating that caspase-9 may act as an initiator caspase in cisplatin-induced apoptosis (23,24).

The activation of the PKCε isoform has been reported to be antiapoptotic in various cellular systems, including lung and prostate cancer cells (25,26). Whereas, its overexpression has been found to inhibit the apoptosis of melanoma (27) and glioma (11) cells. In the current study, the full-length PKCε was found to be particularly small in the two cell lines prior to and following treatment with 5-FU, and was not found to undergo cleavage or phosphorylation following treatment with 5-FU, which indicated that PKCε is not involved in the resistance of CRC to 5-FU-induced apoptosis.

The cleavage and activation of PKCδ has been reported as a response to various apoptotic stimuli, including radiation, oxidative stress and chemotherapeutic agents (13,28). These stimuli may activate the enzyme by phosphorylation and cleavage (26). In the present study, 5-FU was found to induce the activation of PKCδ in HCT116 cells, however, not in the SW480 cell line.

The kinetics of PKCδ activation following treatment with 5-FU in CRC cells was studied by western blot analysis using specific antibodies against PKCδ, as well as p-PKCδ (F-7) that targets the phosphorylated Thr 505 of PKCδ (the activation loop motif), and p-PKC (Ser 645), which targets the phosphorylated Ser 645 of PKCδ (the turn motif). The phosphorylation of PKCδ in the activation loop preceded that which occurred at

the turn motif and was downregulated. Similarly, inactivation of PKCδ implied the initial dephosphorylation at Thr 505 prior to that at Ser 645. In the present study, it is hypothesized that PKCδ activation following treatment with 5-FU may occur in a stepwise manner, initially requiring phosphorylation of the activation loop, followed by phosphorylation of the turn motif.

Although the current study demonstrates that the specific phosphorylations of PKCδ at the sites of the activation loop and the turn motif (critical steps prior to the effective activation of the protein), previous studies have indicated that these phosphorylations are not a prerequisite for the enzymatic activity of PKCδ (29), and that the only structural modification enabling PKCδ to exert its proapoptotic activity is its cleavage to the fully active 40-kDa catalytic fragment (CF) (30). In HCT116 cells, the current study showed that 5-FU increased the expression of full-length PKCδ and induced the cleavage of the enzyme to its CF. These findings may indicate that 5-FU induces PKCδ activation in CRC cells.

PKCδ promotes apoptosis by acting on different signaling pathways, including the mitogen-activated protein kinase (MAPK) signaling pathway and its members; p38 (31), extracellular signal-regulated kinase (32) and c-Jun N-terminal kinase (13). Furthermore, previous studies have shown that the c-Abl-PKCδ-p38 MAPK signaling pathway may trigger the mitochondrial apoptotic signaling pathway by activating the proapoptotic Bcl-2 proteins, Bax and Bak (33,34). Furthermore, caspase-9 is activated during the early stages of the intrinsic apoptotic signaling pathway, immediately following the formation of the apoptosome, although predominantly prior to all other caspases (35). Therefore, in the present study it was hypothesized that when there is a cross-link between the 5-FU-induced PKCδ activation and the caspase-dependent apoptosis that is induced by 5-FU in CRC cells, it may be via the c-Abl-PKCδ-p38 MAPK signaling pathway.

In conclusion, the results of the present study indicate that PKCε is not involved in the resistance of CRC cells to 5-FU chemotherapy, and that 5-FU may induce apoptosis by activating PKCδ and caspase-9. Our ongoing studies will be directed towards elucidating the exact sequence of events, which are triggered following the activation of PKCδ by 5-FU in CRC and to evaluate the signaling of caspase-9 activation.

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